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Xenopus p21-activated kinase 5 regulates blastomeres adhesive properties during convergent extension movements

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Abstract

The p21-activated kinase (PAK) proteins regulate many cellular events including cell cycle progression, cell death and survival, and cytoskeleton rearrangements. We previously identified X-PAK5 that binds the actin and microtubule networks, and could potentially regulate their coordinated dynamics during cell motility. In this study, we investigated the functional importance of this kinase during gastrulation in *Xenopus*. X-PAK5 is mainly expressed in regions of the embryo that undergo extensive cell movements during gastrula such as the animal hemisphere and the marginal zone. Expression of a kinase-dead mutant inhibits convergent extension movements in whole embryos and in activin-treated animal cap by modifying behavior of cells. This phenotype is rescued in embryo by adding back X-PAK5 catalytic activity. The active kinase decreases cell adhesiveness when expressed in animal hemisphere and inhibits the calcium-dependent reassociation of cells, while dead X-PAK5 kinase localizes to cell–cell junctions and increases cell adhesion. In addition, endogenous X-PAK5 colocalizes with adherens junction proteins and its activity is regulated by extracellular calcium. Taken together, our results suggest that X-PAK5 regulates convergent extension movements in vivo by modulating the calcium-mediated cell–cell adhesion.

Keywords: X-PAK5; *Xenopus* embryogenesis; Cell adhesion; Convergent extension

Introduction

During gastrulation, germ layers undergo temporally and spatially coordinated movements that bring progenitor cells into positions from which they later form tissues and organs. In *Xenopus*, convergence extension (CE) movements are essential early during development for the formation and the elongation of the body axis. During CE movements, cells of the marginal zone polarize, elongate, and align before they intercalate between one another (Keller et al., 2000; Shih and Keller, 1992). These lateral cell movements result in the mediolateral narrowing (convergence) and anteroposterior lengthening (extension) of the embryo.

In *Xenopus* embryo, Wnt signaling is best known as a regulator of dorsal cell fate through a « canonical » β-catenin pathway. Wnt also tightly regulates CE movements through activation of two « noncanonical » pathways that respond to members of Wnt superfamily ligands such as Wnt-5a and Wnt-11 (Tada and Smith, 2000; Torres et al., 1996). These ligands, respectively, turn on the Wnt/Ca^{2+} and the Wnt/JNK pathways (the vertebrate equivalent to the planar cell polarity or PCP pathway in *Drosophila*) (for review, see Kuhl, 2002; Tada et al., 2002; Wallingford et al., 2002) that act as antagonists of the Wnt/β-catenin signaling (for review, see Weidinger and Moon, 2003). In response to noncanonical Wnt signaling, cells eventually develop the capacity to change their shape, their polarity,

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and their adhesive properties, thus allowing CE movements to occur.

Indeed, the family of Rho GTPases that controls cytoskeleton reorganization and cell adhesion (for review, see Hall and Nobes, 2000; Jaffer and Chernoff, 2004; Kaibuchi et al., 1999; Schmitz et al., 2000) is also essential for regulating CE movements in *Xenopus* embryo. Inactivation of either endogenous Rac, Cdc42, or RhoA prevents CE movements to occur (Choi and Han, 2002; Habas et al., 2001, 2003; Tahinci and Symes, 2003). Both RhoA and Rac are effectors of the PCP pathway (Habas et al., 2001, 2003) although their activation is not dependent upon each other (Habas et al., 2003). Cdc42 is involved in Wnt/Ca^{2+} signaling and regulates CE movements downstream of Gβγ (Penzo-Mendez et al., 2003) and PKC (Choi and Han, 2002; Penzo-Mendez et al., 2003). While clearly GTPases are involved in CE, the GTPase effectors that regulate changes in polarity, protrusive, and adhesive activities of cells during CE are not well characterized. Changes in polarity and directional movements could involve effectors that capture and stabilize microtubules near the cell cortex such as IQGAP1 and Par6 downstream of Cdc42 or mDia downstream of RhoA (Etienne-Manneville and Hall, 2001; Fukata et al., 2002; Palazzo et al., 2001). Changes in adhesive properties, morphology, protrusive activity, and generation of forces for cell translocation and movements involve reorganization of the actin cytoskeleton and the adherence junctions. A number of GTPase effectors downstream of Rac and Cdc42 could be involved. p21-activated kinases (PAKs) are good candidates for this role since they have been shown to control cell shape and motility (for review, see Bokoch, 2003; Jaffer and Chernoff, 2002). PAK subgroups I and II are distinguished based upon their structural organization and regulation. Binding of GTP-bound Rac or Cdc42 to subgroup I PAKs (PAK1–3) causes the dissociation of the PAK kinase autoinhibitory domain (AID) and the C-terminal catalytic domain, thus allowing autophosphorylation and full activation of the kinase (Buchwald et al., 2001; Chong et al., 2001; Lei et al., 2000). In contrast, subgroup II PAKs (PAK4–6) appear to lack a classic AID and are not directly activated by binding of active Rac and Cdc42 GTPases. Both subgroup kinases are involved in the regulation of MAP kinase cascades, cell cycle, and apoptosis (for review, see Bokoch, 2003; Jaffer and Chernoff, 2002), and were shown to regulate cell cytoskeletal changes. Individual PAKs act through a number of targets including the myosin light chain kinase (MLCK) (Sanders et al., 1999), regulatory myosin light chain (Goeckeler et al., 2000), Caldesmon (Foster et al., 2000), filamin (Vadlamudi et al., 2002), desmin (Ohtakara et al., 2000), Lim kinase (Dan et al., 2001; Edwards et al., 1999), and integrin alpha v beta 5 (Zhang et al., 2002). Subgroup I and II PAKs may carry out different functions in a same cell as highlighted in Schneeberger and Raabe (2003). In developing photoreceptor cells, *Drosophila* subgroup II PAK Mbt depends upon Cdc42 binding for its localization to adherence junctions (Schneeberger and Raabe, 2003) and is required for cell morphogenesis, while DPAK, a subgroup I PAK, is required in growth cones to control axon guidance (Hing et al., 1999).

Human PAK4, a member of subgroup II, induces loss of cell adhesion and anchorage-independent growth that characterize oncogenic cell transformation (Callow et al., 2002; Qu et al., 2001). We previously described a subgroup II *Xenopus* PAK, X-PAK5, that is closely related to hPAK4. X-PAK5 binds the actin and microtubule networks (Cau et al., 2001), and binding of active GTPases to the endogenous kinase induces its relocalization to actin-rich structures. Both the actin and microtubule networks are important in the regulation of cell motility. This led us to investigate whether X-PAK5 may regulate the extensive cell rearrangements that occur during gastrulation in *Xenopus*. In this report, we show that X-PAK5, which is expressed during early development, is present in regions where morphogenetic movements are induced at the onset of gastrulation. Expression of kinase-dead mutant impairs CE movements and results in defects in blastopore closure. Kinase-dead mutants act at least by strengthening cell–cell junctions in embryonic cells while active X-PAK5 changes the adhesive properties of cells. Changes in cell adhesion eventually lead to embryos that develop tissue protrusions. Finally, our data indicate that the catalytic activity of X-PAK5 is regulated upon calcium-induced aggregation of dissociated cells. We finally show that endogenous, active, and dead-kinase mutants of X-PAK5 colocalize at least partially with adherens junction proteins.

Materials and methods

Embryo manipulation

Eggs were collected from *Xenopus* females and artificially fertilized as previously described (Watanabe and Whitman, 1999). Embryos were dejellied before the first cleavage in 3% cysteine and injected in 3% Ficoll in 0.1× Marc's modified Ringer medium (MMR). Embryos were staged according to Nieuwkoop and Faber (1967). Animal caps were dissected at stage 8.5 and incubated in 0.7× MMR in the presence or absence of partially purified human activin A at a final concentration of 8 units/ml (Cooke et al., 1987) (kindly provided by J.C. Smith). Animal caps were cultured to stage 18 equivalent. Cycloheximide treatment was performed by transferring embryos to 0.1 mg/ml cycloheximide in 0.7× MMR.

Anti-X-PAK antibodies

The polyclonal anti-X-PAK5 antibodies have been raised against the unique 122–224 amino acids of X-PAK5 (Cau et al., 2001). Immunopurified antibodies specifically detect a single band of 75 kDa in *Xenopus* eggs and embryos.

PhosphoPAK4/PAK5/PAK6 antibodies (Cell Signalling Technology) were made against a phospho-peptide surrounding the Serine 533 (Ser533) in X-PAK5 equivalent to the Ser474 in hPAK4, the Ser602 in hPAK5, and the Ser560 in hPAK6, in the catalytic loop of the kinase. Phosphorylation at this site was previously shown to be required for full activation of subgroup II PAK kinases (Gnesutta et al., 2001), and these antibodies recognize the activated forms of human PAK4, PAK5, and PAK6 (data not shown). Their characterization in *Xenopus* is presented in Supplementary Fig. S1. Anti-PhosphoPAK4/PAK5/PAK6 antibodies specifically detect autophosphorylated active X-PAK5 kinase and do not cross react with the nonactive form of the kinase. In some Western blot analyses, a second species migrating around 115–120 kDa was recognized as well, preventing the use of these antibodies in immunohistochemistry analyses. However, as X-PAK5 is the only *Xenopus* PAK kinase of the subgroup II identified so far and because X-PAK5 is the major endogenous protein detected in *Xenopus* embryo, we will refer for simplicity to the endogenous PhosphoPAK4/PAK5/PAK6 signal detected in *Xenopus* embryos as phosphoPAK5 signal even though other X-PAKs from subgroup II may also be detected by these antibodies. *Xenopus* X-PAK2 polyclonal antibodies have been raised by immunization of rabbits with the recombinant N-terminal domain of X-PAK2 (amino acids 2–214) in fusion with the MBP and immunopurified by using the corresponding recombinant protein (Cau et al., 2000).

Western blot analyses, immunoprecipitations, and kinase assays

Western blot analyses were performed essentially as previously described (Faure et al., 2000). Whole embryos or animal caps were homogenized (10 μl per embryo and 10 μl for 10 caps) in lysis buffer (20 mM Tris pH 8, 50 mM NaCl, 50 mM NaF, 10 mM βglycerophosphate, 2 mM EDTA, 1% NP 40, 20 mM aprotinin, 0.75 mM PMSF). Lysates were centrifuged at 14,000 × *g* for 10 min at 4°C; supernatants were analyzed on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose (Protran). Membranes were blocked at room temperature for 1 h in 2% milk in TBST (10 mM Tris pH 8, 150 mM NaCl, 0.2% Tween 20) and incubated overnight at 4°C with either immunopurified anti-X-PAK5 antibody (abn122) or anti-X-PAK2 antibody (Cau et al., 2000, 2001) or anti-PhosphoPAK4/5/6 antibodies (Cell Signalling Technology) or anti-β tubulin (clone E7, Developmental Studies Hybridoma Bank, DSHB, University of Iowa) or anti-γ tubulin antibody (SIGMA). After several washes in TBST, membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (Amersham Biosciences), washed in TBST, and developed with cheminumilescence reagents (Perkin-Elmer, Life Sciences) as described by the manufacturer. Immunoprecipitation experiments were performed essentially as previously described (Cau et al., 2000). One equivalent embryo lysate was diluted in TLB buffer (20 mM Tris pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM orthovanadate) and incubated for 2 h with the immunopurified anti-X-PAK5 antibody (abn122). For kinase assays, immunoprecipitates were washed twice in TLB buffer and twice in kinase buffer (25 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 25 mM βglycerophosphate, 2 mM DTT, 0.1 mM orthovanadate) and then incubated for 15 min in 20 μl of kinase buffer containing 2 μl of histone H2B at 5 mg/ml, 1 mM ATP, and 0.5 μl of γ-^{32}P(ATP) (3000 Ci/mmol). Immunoprecipitates were subjected to 20% SDS-PAGE and phosphorylated-histone H2B visualized by autoradiography.

Immunohistochemistry and immunofluorescence analyses

Xenopus embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA pH 8, 1 mM $MgSO_4$, 3.7% formaldehyde) for 2 h at room temperature and analyzed by immunochemistry analysis as previously described (Faure et al., 2000). Incubation with the immunopurified anti-X-PAK5 antibody (abn122) at a dilution of 1/100 in TBST was carried out overnight at 4°C. After several washes, embryos were incubated with peroxidase-conjugated secondary antibody (Amersham Biosciences). Signal was detected by using the Fast 3,3'-Diaminobenzidine Tablet sets (SIGMA). For confocal analyses, uninjected animal cap explants were dissected at stage 8 and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. After fixation, explants were incubated overnight with anti-X-PAK5 antibody (abn122) in PBS supplemented with 0.05% Triton X-100 and 5% fetal calf serum (PBSTF). After washes with several batches of TBSTF, explants were incubated with anti-rabbit antibodies linked to Alexa 670 (Molecular Probes) and observed with LSM510Meta confocal microscope. Alternatively, cap explants dissected from GFP-X-PAK5-injected embryos were mounted in dissection buffer and immediately observed after dissection to avoid diffusion of the gfp signal.

Epithelial *Xenopus* A6 cells were used for transfection experiments. Cells were transfected for 24 h by either GFP-X-PAK5 K/R or GFP-X-PAK5/EN mutants in pCS2+ vector using Fugene reagent (Roche) following the manufacturer's recommendations. Transfected and mock-transfected cells were fixed with 100% methanol for 8 min at −20°C, incubated with either E-cadherin (clone 5D3, Developmental Studies Hybridoma Bank, DSHB, university of Iowa) or γ-catenin (BD transduction laboratories) monoclonal antibodies. In addition, mock-transfected cells were incubated with Abn122. Primary antibodies were used for 1 h at room temperature in PBSTF, then cells were extensively washed in PBSTF and incubated with anti-mouse Alexa 555 and anti-rabbit Alexa 670 (Molecular Probes). Following extensive washes in PBSTF, cells were mounted in Vectashield and observed with LSM510Meta confocal microscope.

β-Galactosidase staining

β-Galactosidase (β-gal) staining was carried out essentially as described (Tada et al., 1997). Embryos were fixed in MEMFA at 4°C for 2 h. Then, they were washed in PBS containing 0.02% NP 40 and stained at 20°C in a PBS solution containing 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 Mm $k_4Fe(CN)_6$, 2 mM $MgCl_2$, and 0.02% NP 40.

In vitro transcription

We previously isolated the full-length X-PAK5 cDNA and subcloned it in pEGFP vector (Cau et al., 2001). The cDNA encoding for a GFP-X-PAK5 fusion was subcloned in pCS2 + vector. X-PAK5 K/R, X-PAK5S533A, and X-PAK5/EN mutants were constructed by site-directed mutagenesis. The following changes were made: K to R at residue 409 located in the putative ATP binding site for X-PAK5 K/R, S to A at residue 533 in the activation loop of X-PAK5 kinase for X-PAK5S533A. When expressed either in cells or in embryos, both X-PAK5 K/R and X-PAK5S533A mutants display no kinase activity. For X-PAK5/EN mutant, serine residues at position 504 and 533 have been substituted, respectively, by E and N (Cau et al., 2001). All mutations were verified by sequencing. The pCS2 X-PAK5 constructs were linearized with Nsi1 restriction enzyme for in vitro transcription. Capped-synthetic mRNAs were generated with SP6 polymerase by using the mMessage mMachine kit (Ambion) as described by the manufacturer.

In situ hybridization

Whole-mount in situ hybridization was performed essentially as described (Harland, 1991). *XBrachyury* (*Xbra*), *Goosecoid* (*gsc*) (Cho et al., 1991; Smith et al., 1991), and *Xotx-2* (Pannese et al., 1995) probes were kindly provided by Dr. J. C. Smith, and *Xnot* probe (Hoppler and Moon, 1998; von Dassow et al., 1993) by Dr. R. Moon.

Cell adhesion assay

Cell adhesion assay was carried out as previously described (Choi and Han, 2002). Briefly, embryos were injected at two-cell stage into the animal pole; animal caps were dissected at stage 8.5 and dissociated in Ca^{2+}/Mg^{2+}-free medium (CMFM) (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris pH 7.6) until dissociation was complete. Dissociated cells were reaggregated at 20°C in CMFM plus 4 mM Ca^{2+} with gentle shaking and then fixed in MEMFA at the indicated time.

Procaspase 3 cleavage assay

The procaspase 3 cleavage assay was performed essentially as described by Hensey and Gautier (1997). Briefly, five embryos were homogenized in EB buffer (80 mM β-glycerophosphate pH 7.3, 15 mM $MgCl_2$, 20 mM EGTA, 10 mM DTT plus protease inhibitors). Homogenates were centrifuged at 14,000 × *g* for 10 min at 4°C. Three microliters of supernatant was mixed with 1 μl of (^{35}S)methionine-labeled mammalian procaspase 3 and incubated at room temperature for 20 min. Samples were run on a 15% SDS-PAGE gel and transferred to nitrocellulose (Protran). Cleavage of procaspase 3 was visualized by autoradiography. In vitro translation of pcDNA3 procaspase 3 was performed as previously reported (Faure et al., 1997).

Results

Spatiotemporal expression pattern of X-PAK5 during early Xenopus development

We first examined the temporal expression pattern of X-PAK5 during early *Xenopus* embryogenesis by Western blot analysis using specific antibodies to X-PAK5 (Cau et al., 2001). X-PAK5 is expressed from maternal mRNAs up to midblastula transition (MBT) (Fig. 1A, upper panel). Levels of X-PAK5 expression increase during the course of gastrulation when zygotic expression starts (between stages 9 and 10) (Fig. 1A, bottom panel) and becomes constant later in development. This pattern is specific to X-PAK5 as expression of X-PAK2, another X-PAK member, is constant throughout early embryogenesis (Fig. 1A).

We then examined the localization of X-PAK5 across the animal–vegetal axis. We dissected animal (A), marginal (M), and vegetal (V) regions from whole embryos at early gastrula stage and extracts were analyzed by Western blot using X-PAK5 antibodies as in Fig. 1A. With respect to the β-tubulin Western blot control (see legend of the figure), stage 10 animal and marginal explants contain similar levels of X-PAK5 while its expression level is lower in the vegetal region (Fig. 1B). By contrast, X-PAK2 expression level is similar in the three different dissected regions of the embryos.

We then visualized the localization of X-PAK5 during gastrulation by immunochemistry analysis (Fig. 1C). At the onset of gastrulation (stages 10+ and 10.25), X-PAK5 expression is essentially located in the animal pole and the marginal zone as previously shown by Western blot analysis (Fig. 1B). In the marginal zone, the X-PAK5 signal is detected in mesodermal-fated cells that migrate inwards the embryo. Later during gastrulation (stage 11.5), X-PAK5 expression is detected in the ectoderm and in involuting dorsal and ventral mesoderm. Taken together, our results indicate that the X-PAK5 protein is expressed primarily in the cells of both the animal hemisphere and the marginal zone where extensive cell rearrangements take place during gastrula.

We further analyzed the localization of X-PAK5 in embryonic cells from animal cap explants dissected at stage 8 and from dorsal marginal zone (DMZ) explants dissected at stage 10.25 using a confocal microscope (Fig. 1D). In

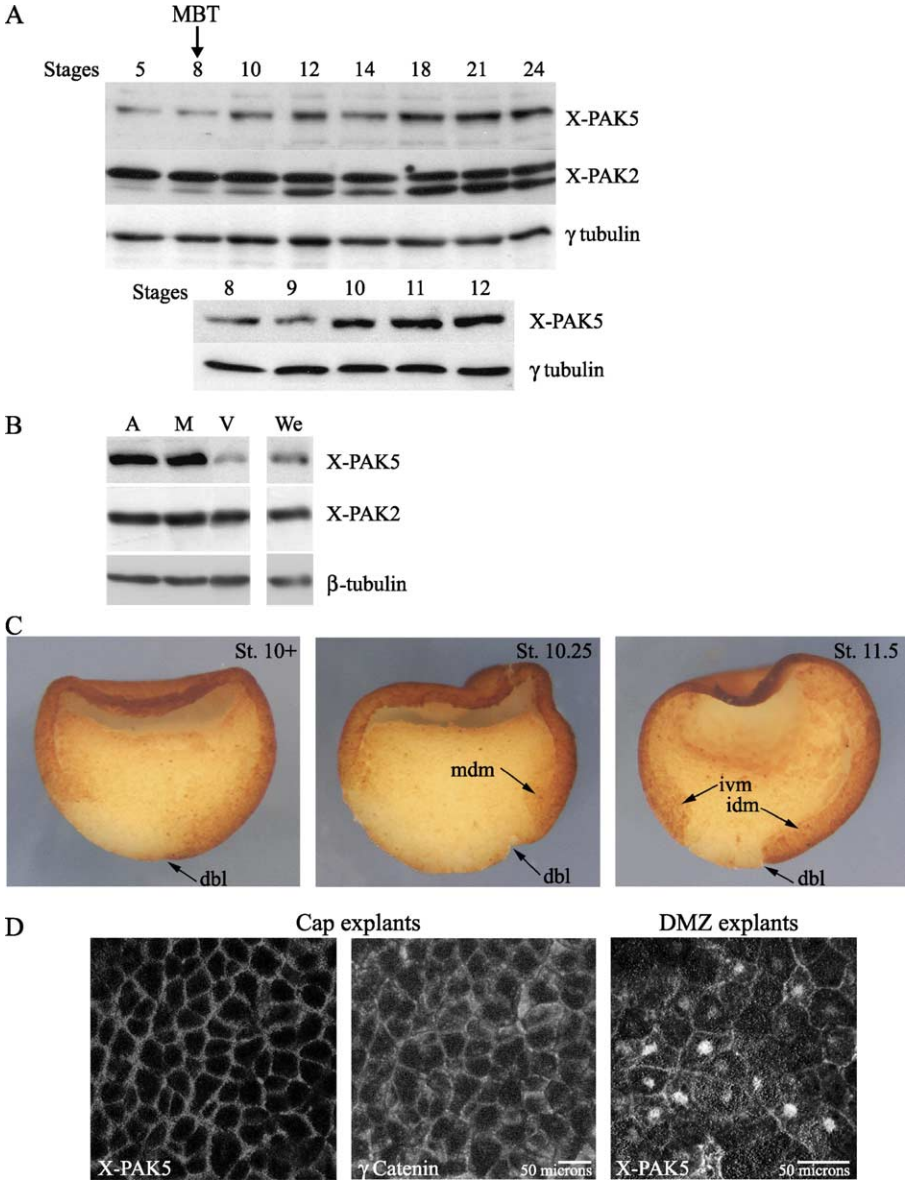


Fig. 1. Spatiotemporal expression pattern of X-PAK5 during early *Xenopus* development. (A) Temporal expression pattern of X-PAK5. Protein extracts from embryos harvested at the indicated stages were subjected to Western blot analyses with anti-X-PAK5, anti-X-PAK2, and anti-γ tubulin antibodies. Levels of γ-tubulin reflect proteins loading. (Upper panel) Temporal expression pattern of X-PAK5 during early development (from stage 5 to stage 24). (Bottom panel) Temporal expression pattern of X-PAK5 from blastula to gastrula. Level of X-PAK5 increases during the course of gastrulation when zygotic expression starts and levels remain constant from stage 10 until at least stage 24. In control, temporal expression of X-PAK2 is shown. Note that X-PAK2 antibodies reveal a doublet in embryonic lysates and the signal associated with the bottom band increases during the course of development. The same pattern is observed during early development if antibodies directed against the C-terminal region of X-PAK2 are used for Western blot detection suggesting that the bottom band is not a proteolytic product of X-PAK2. This lower band is not detected in *Xenopus* oocytes (Cau et al., 2000). However, it is repeatedly detected around stage 9 during development and is likely due to either a posttranslational modification of X-PAK2 or to the expression of a zygotic closely related X-PAK2 isoform whose expression increases after MBT. MBT, midblastula transition. (B) Spatial distribution of X-PAK5 across the animal–vegetal axis. *Xenopus* embryos were dissected at stage 10^{+} into animal poles (A), equatorial marginal zones (M), and vegetal regions (V). Control whole embryos (We) were harvested at the same stage and embryonic lysates performed. Since cell size varies greatly across the animal–vegetal axis, protein extracts were standardized by Western blot analysis using β-tubulin as a loading control for cellular volume. Western blot analyses of similar volumes of dissected pieces and whole embryos (with respect to the β-tubulin control) are shown. X-PAK5 protein is mainly localized in the animal pole and the equatorial marginal zone in the early gastrula embryo. (C) Immunostaining of X-PAK5 across the dorsoventral axis. *Xenopus* embryos were fixed in MEMFA at the onset of gastrula (stage 10+), at midgastrula (stage 10.25), and late gastrula (stage 11.5), and bisected along the dorsoventral axis for immunohistochemistry. Dorsoventral orientation was determined by the dorsal blastopore lip. X-PAK5 expression is essentially located in the ectoderm and in marginal zone cells including the mesodermal-fated cells that migrate inwards the embryo. Some staining was also detected in nuclei of endodermal cells. No significant signal was detected in immunohistochemical control detection when primary antibody addition was omitted. (D) Subcellular localization of endogenous X-PAK5 in animal explants (dissected at stage 8) and dorsal marginal zone (DMZ) explants (dissected at stage 10.25) using anti-PAK5 Abn122. Immunodetection of γ-catenin on cap explants serves as a control for cell–cell junction staining. Abbreviations: mdm, migrating dorsal mesoderm; dbl, dorsal blastopore lip; ivm, involuting ventral mesoderm; idm, involuting dorsal mesoderm.

animal cap cells, some cytoplasmic staining of X-PAK5 is detected, but the endogenous kinase is mainly localized to the cytoplasmic membrane and at cell–cell junctions where it partially colocalizes with γ catenin, an adherens junction and desmosome protein component (Ben-Ze'ev and Geiger, 1998). Occasionally, some staining is detected in nuclei as well (data not shown). In DMZ cells, X-PAK5 is also localized at cell–cell junctions and some nuclear and cytoplasmic staining is also detected.

Expression of a kinase-dead mutant of X-PAK5 affects CE movements during gastrulation

We showed that X-PAK5 is expressed during early development both when and where extensive cell movements take place (Fig. 1). In order to study the role of X-PAK5 during early development, we attempted to deplete X-PAK5 via a morpholino antisense oligonucleotide (MO). However, injection of the X-PAK5 MO failed to reduce X-PAK5 protein level (data not shown). To alleviate this problem, we took advantage of a full-length kinase-dead X-PAK5 mutant in fusion with the green fluorescent protein (GFP-X-PAK5 K/R) (Cau et al., 2001) that we will refer in the text as X-PAK5 K/R for simplicity. Injection of X-PAK5 K/R mRNA in the DMZ of four-cell stage embryos induces profound developmental defects in a dose-dependent manner whereas the development of gfp-injected control embryos is normal. At high doses (500–900 pg mRNAs injected per embryo), X-PAK5 K/R expression causes strong developmental defects that are clearly seen at stage 6–7 around the presumptive injection site. The majority of these embryos does not survive beyond stages 10–11. Indeed, the injected cells appear both larger than their noninjected neighboring cells and delayed in their division (data not shown).

Subsequently, in this study, we always used lower doses of mRNAs (100–400 pg per embryo). The expression level of the injected X-PAK5 K/R mutant is shown in Fig. 2B. Under these conditions, most of the X-PAK5 K/R-injected embryos examined at stage 35 develop dorsal defects that are characterized by a shortened anteroposterior axis, a strong dorsal flexure compared to gfp-injected embryos (Fig. 2A, compare panel a to b), and, in some cases, an open neural tube (see arrow in Fig. 2A, panel c). As such a phenotype may result from defects in cell movements early during gastrulation, we analyzed the effect of X-PAK5 K/R expression at earlier stages during development (Fig. 2C and Table 1). During early development, X-PAK5 K/R-injected embryos appear normal as they form the dorsal blastopore lip at the same time as the sibling embryos (data not shown). However, at stage 11, by the end of gastrulation, 80% ($n = 75$) of X-PAK5 K/R-injected embryos display a large-sized blastopore compared to controls (Fig. 2C, compare panels a and b) that will eventually raise two phenotypes. The most severe phenotype is characterized by an opened blastopore at stage 12 by comparison with controls (Fig. 2C, compare panels c and d). When they reach neurula stages, these embryos present a mass of noninternalized yolk resulting from a defect in mesoderm involution during gastrulation (Fig. 2C, compare panels e and g to f and h). Finally, these embryos will develop in 39% of cases ($n = 75$) a strong dorsal flexure and an open neural tube as shown previously (Fig. 2A, panel c). In the less severe phenotype, the closure of the blastopore is delayed and the neural folds fail to fuse correctly; at neurula stages, these embryos will develop in 46% of cases ($n = 75$) the bent axis shown in Fig. 2A (panel b).

These results indicate that expression of X-PAK5 K/R is likely to interfere with cells movements that occur during gastrulation. In order to confirm this hypothesis, we analyzed over time the behavior and localization of cells that express gfp control or X-PAK5 K/R proteins by following the gfp fluorescent emission of injected living embryos. Prior to the onset of cell movements in early gastrulae, fluorescent cells are located at the level of injection site in the DMZ both in gfp and in X-PAK5 K/R-injected embryos (data not shown). In stage 12 control embryos, the gfp-injected cells have moved toward the midline of the embryo as a result of cell intercalation; they are positioned from the DMZ, the site of injection, to the blastopore lip through which they are moving inside the embryo (Fig. 2C, panel i). However, in X-PAK5 K/R-injected embryos at stage 12 equivalent, expressing cells remain clustered at the level of the injection site in the DMZ; they do not rearrange into the rod-like structure observed in controls, indicating that they fail to intercalate (Fig. 2C, panel j). This is confirmed by gfp stainings later in development at neurula stage (Fig. 2C, compare panels k to l). Thus, these results further indicate that CE movements are impaired in X-PAK5 K/R-injected embryos. To confirm that X-PAK5 functions in regulating CE, we used the animal cap assay. This assay is widely used since it mimics in vitro the CE movements that take place in vivo (Symes and Smith, 1987). Gfp and X-PAK5 K/R mRNAs were microinjected in the animal pole of two-cell stage embryos and animal cap dissected at blastula stage (stage 8.5). Explants were then cultured in the presence or absence of activin until stage 18 equivalent (Fig. 2D). In the absence of activin treatment, both gfp control and X-PAK5 K/R caps form tight balls of cells. As expected, in response to activin, caps of gfp-injected embryos elongate. In contrast, expression of X-PAK5 K/R strongly inhibits the elongation of animal explants induced by activin. Taken together, our results show that expression of a kinase-dead mutant of X-PAK5 inhibits CE movements that take place during gastrulation in whole embryo and in activin-treated animal caps.

Expression of X-PAK5 K/R affects CE movements during gastrulation without preventing mesoderm specification

X-PAK5 K/R could affect CE movements either by acting directly on cell shape and migratory behavior or

alternatively by modifying tissue-specific gene expression. In order to distinguish between these two possibilities, we analyzed by in situ hybridization the expression pattern of the mesodermal markers *Brachyury* (*Xbra*), *Goosecoid* (*Xgsc*), *Xotx-2*, and *Xnot*. During early development, these markers are all expressed in cells that undergo CE movements and that will form the axial mesoderm. If X-PAK5 directly regulates cell movements, expression of X-PAK5

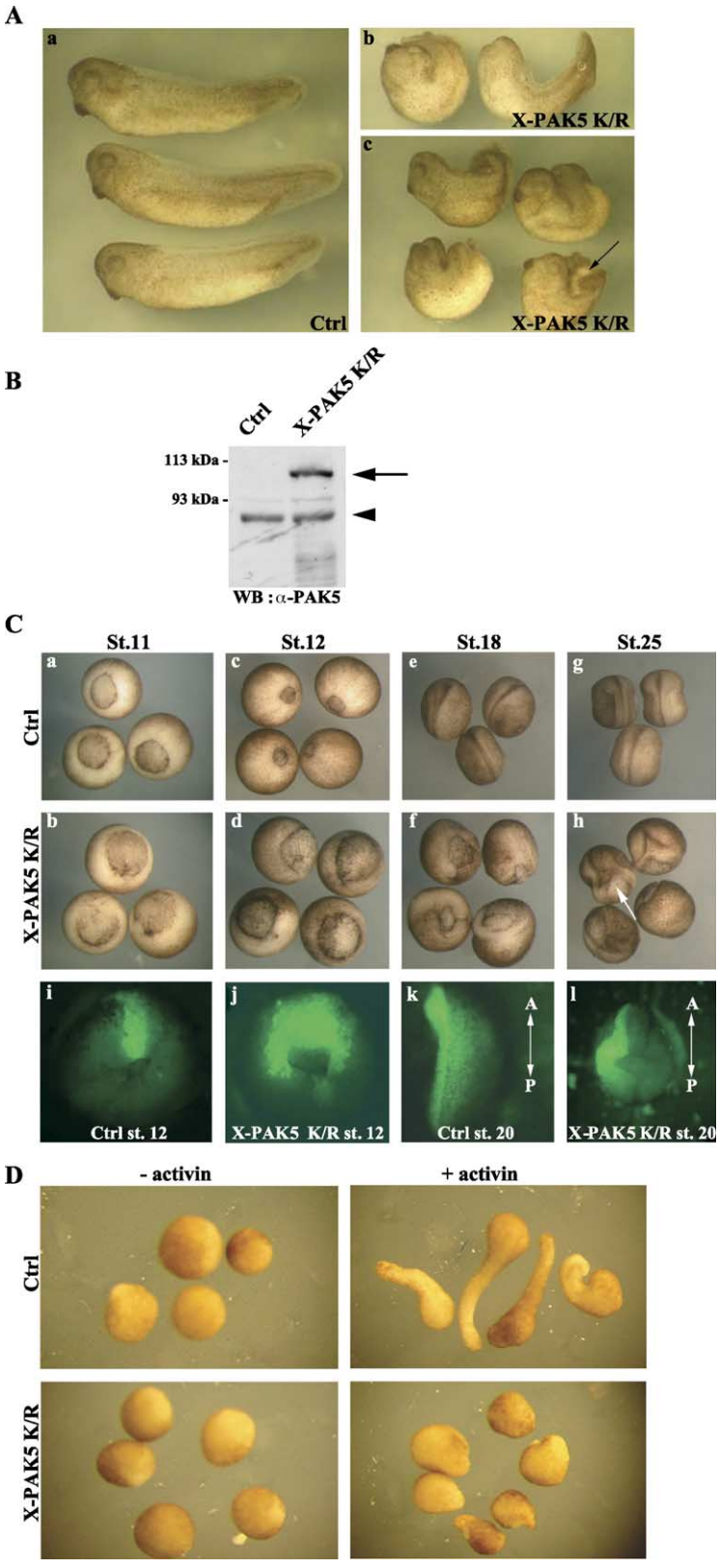


Table 1
Effects of X-PAK5 K/R overexpression on blastopore closure and early development

mRNA injected	*n*	Blastopore		Late phenotypes		
		Normal	Open	Normal	Open neural tube	bent axis
Gfp	70	93	7	91	3	6
X-PAK5 K/R	75	20	80	15	39	46

Embryos were injected at four-cell stage in the DMZ with indicated mRNAs. Blastopore closure and late phenotypes were scored at stage 13 and 23, respectively. Numbers refer to percentages of phenotypes, *n* refers to the total number of embryos analyzed.

K/R mutant should not affect expression of these genes and specification of tissues. Early during gastrulation, *Xgsc* is expressed in the deep layer of the upper part of the dorsal blastopore lip and later restricted to the notochord and prechordal mesoderm (Cho et al., 1991). *Xotx-2* expression is first detected in migratory deep zone cells that are fated to give rise to prechordal mesoderm and later in the presumptive anterior neuroectoderm (Pannese et al., 1995). During gastrulation, *Xbra* is first detected in the entire marginal zone and later becomes restricted to the blastopore and the developing notochord (Smith et al., 1991). It was reported that interfering with receptor tyrosine kinase (RTK) signaling that regulates mesoderm specification prevents *Xbra* expression (Amaya et al., 1993; Myers et al., 2004). Here, we show that interfering with X-PAK5 signaling does not affect *Xbra* expression level at the onset of gastrulation, as similar levels of *Xbra* are detected in X-PAK5 K/R-injected and control embryos (Fig. 3A). Furthermore, at stage 10+, *Xgsc* and *Xotx-2* are identically expressed in X-PAK5 K/R and control embryos as well (Fig. 3B and data not shown). Altogether, our results show that X-PAK5 K/R expression does not interfere with the expression of mesodermal markers. However, as development proceeds to late gastrula/early neurula stages, the distribution of mRNAs coding for these mesodermal genes is affected in X-PAK5 K/R-injected embryos consistent with a role of X-PAK5 K/R in modifying the behavior of DMZ cells. At stage 11.5, the expression domain of *Xbra* is broader around the blastopore but reduced in notochordal mesodermal cells (see black arrow in Fig. 3C), indicating that mesodermal cells failed to involute properly during gastrulation. A similar distribution of *Xbra* staining at stage 12 was previously observed in embryos expressing mutants of CDC42 and Xfz7 in which CE movements are impaired (Choi and Han, 2002; Djiane et al., 2000). At stage 12, *Xgsc* expression is localized at the dorsal blastopore lip and in the anterior region of the embryo (arrows in Fig. 3D) while its expression is restricted to the anterior embryonic domain in controls. Expression of *Xotx-2* in X-PAK5 K/R-injected late gastrulae (stage 12.5) is detected in a broad domain that extends more dorsovegetally than in control embryos (Fig. 3E). In some X-PAK5 K/R-injected embryos, expression of *Xotx-2* is also detected near the blastopore lip (see arrows in Fig. 3E). Finally, we analyzed in early neurula embryos the expression of *Xnot* that was reported to be localized to the developing notochord by the end of gastrulation (von Dassow et al., 1993). While expression of *Xnot* is detected in the notochord in early neurula control embryos, its expression is observed in the dorsal blastopore lip in X-PAK5 K/R-injected embryos at equivalent developmental stage, indicating that the dorsal mesoderm failed to involute and notochord was not elongated in these embryos (Fig. 3F). Altogether, our results indicate that interfering with X-PAK5 signaling in the DMZ modifies the behavior of cells that undergo CE movement.

Rescue of X-PAK5 K/R phenotype by expression of X-PAK5 catalytic activity

We showed that X-PAK5 K/R impedes CE movements during gastrulation. This suggests that the kinase activity of X-PAK5 may be required for CE movements to occur. In order to confirm this hypothesis, we supplemented embryos with exogenous kinase activity by injecting various amounts of mRNA coding for constitutively active X-PAK5 (X-PAK5/EN) (Cau et al., 2001). When injected at high doses (>500 pg mRNAs per embryo) in the DMZ of embryos at four-cell stage, the majority of expressing

Fig. 2. Ectopic expression of X-PAK5 K/R affects gastrulation movements. (A–C) Embryos were injected at four-cell stage in DMZ with either 300 pg of gfp mRNA or with 300 pg of GFP-X-PAK5 K/R mRNA and examined during their development. (A) Representative sample of (a) GFP-injected control (Ctrl) embryos at stage 35. (b–c) GFP-X-PAK5 K/R-injected embryos at stage 35 equivalent. These embryos exhibit a shortened bent anteroposterior axis. In the most severe phenotype (c), the neural tube remains opened as indicated by a black arrow. Note that GFP-X-PAK5 K/R-injected embryos have a fully formed head. (B) Analyses of expression level of GFP-X-PAK5 K/R mutant in *Xenopus* embryos. Anti-X-PAK5 Western blot analyses of extracts from gfp control (Ctrl) and GFP-X-PAK5 K/R-injected embryos harvested at stage 12. Endogenous X-PAK5 protein migrates around 80 kDa (see black arrowhead) whereas GFP-X-PAK5 K/R mutant migrates around 110 kDa (see black arrow). (C) Representative sample of embryos examined during gastrula and neurula stages. (a, c, e, and g) GFP control (Ctrl) embryos. (b, d, f, and h) GFP-X-PAK5 K/R-injected embryos. (a and b) stage 11, (c and d) stage 12, (e and f) stage 18, and (g and h) stage 25. White arrow in panel h indicates the presence of visible mass of endoderm that has not been internalized during gastrula. (i–l) Lineage tracer analyses of convergence extension movements in gfp control and GFP-X-PAK5 KR-injected embryos using gfp-emitted fluorescence. (i) GFP control (Ctrl) embryo at stage 12. (j) GFP-X-PAK5 KR-injected embryo at stage 12 equivalent. The CE movements of injected cells are impaired. (k) GFP control (Ctrl) embryo at stage 20. (l) In stage 20 GFP-X-PAK5 KR-injected embryos, the labeled mesodermal cells did not involute. Anterior (A) and posterior (P) regions of the embryos are indicated in k and l. (D) Animal cap assay. Two-cell stage embryos were injected in the animal pole either with gfp mRNA (300 pg per embryo) or with GFP-X-PAK5 mRNA (300 pg per embryo). Animal cap explants were excised at stage 8.5 and incubated in the presence (+activin) or the absence (−activin) of recombinant activin. In response to activin, animal cap explants from gfp-injected control (Ctrl) embryos elongate while expression of GFP-X-PAK5 K/R inhibits their elongation.

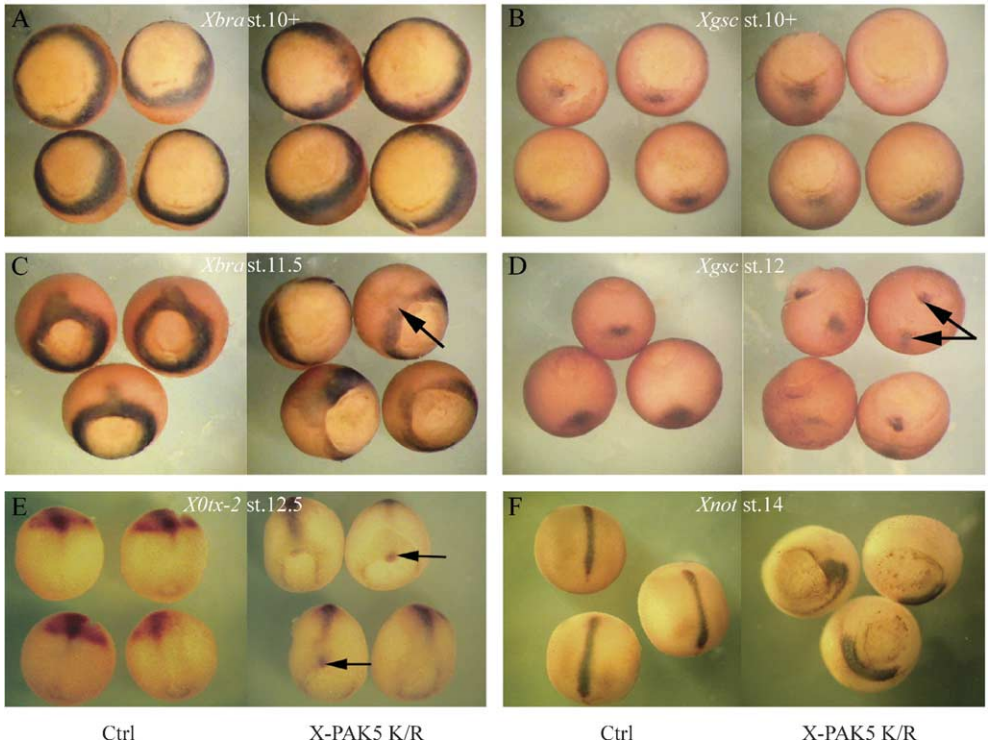


Fig. 3. X-PAK5 K/R expression affects the localization of mesodermal markers. Four-cell stage embryos were injected either with 300 pg of gfp mRNA or with 300 pg of GFP-X-PAK5 K/R mRNA in the DMZ and fixed at the indicated time. Expression of mesodermal genes was analyzed by in situ hybridization. Expressions in gfp control (Ctrl) and GFP-X-PAK5 K/R-injected embryos are compared in each panel. (A) Expression of *Brachury* (*Xbra*) at stage 10+. (B) Expression of *gooscoid* at stage 10+. (C) Expression of *Brachury* (*Xbra)* at stage 11.5. Note that *Xbra* expression in GFP-X-PAK5 K/R embryos is reduced in the notochordal mesoderm (as indicated by black arrow) indicating that in GFP-X-PAK5 K/R-injected embryos, the dorsal mesoderm failed to involute during gastrulation. (D) Expression of *Xgsc* at stage 12. Black arrows indicate the two domains of *Xgsc* expression in GFP-X-PAK5 K/R-injected embryos. (E) Expression of *Xotx-2* at stage 12.5. Black arrows indicate *Xotx*-2 staining in the blastopore lip in X-PAK5-KR-expressing embryos. (F) Expression of *Xnot* at early neurula stage. In gfp control (Ctrl) embryos, *Xnot* expression is localized in the notochordal mesoderm whereas its expression is localized in the dorsal blastopore lip in GFP-X-PAK5 K/R-injected embryos, indicating that the notochord was not elongated in GFP-X-PAK5 K/R-injected embryos.

embryos does not survive beyond stage 10 (data not shown). At lower doses (100–400 pg mRNAs per embryo), all X-PAK5/EN-injected embryos develop lesions around the injection site in the DMZ that affects CE movements during gastrulation in a dose-dependant manner (data not shown). This result indicates that both gain and loss of X-PAK5 function affect CE movements at gastrula. Thus, a tight balance is likely required between active and inactive states in order to regulate CE movements. We thus examined whether the effect of X-PAK5 K/R expression on CE could be rescued by X-PAK5/EN. Because of the high activity of X-PAK5/EN, we first injected different amounts of its mRNA in the DMZ of embryos at four-cell stage in order to establish the minimal activity that would not be deleterious for CE movements (data not shown). Then, we injected this amount of mRNA alone or in combination with X-PAK5 K/R mRNA. We examined both the closure of the blastopore at the end of gastrulation (stage 12) and the late phenotypes (stage 28) of injected and co-injected embryos. In the representative experiment described in Fig. 4 (see also Table 2), X-PAK5 K/R expression (300 pg mRNA per embryo) strongly delays blastopore closure at gastrulation in 74% (n = 80) (Fig. 4B), and 62% (n = 80) of the tadpole embryos develop with a shortened anteroposterior axis and a strong dorsal flexure (Fig. 4F). When injected alone (50 pg mRNAs per embryo), X-PAK5/EN, as mentioned above, had no significant effect on blastopore closure (Fig. 4C) and embryos develop as gfp-injected controls (compare Figs. 4E and 4G). However, injection of 50 pg of X-PAK5/EN mRNA significantly rescues the delay in blastopore closure induced by X-PAK5 K/R (Fig. 4D) and 70% (n = 72) of the co-injected embryos develop as controls (Fig. 4H). In conclusion, the kinase-dead X-PAK5 phenotype is rescued by injection of X-PAK5/EN, indicating that a specific amount of X-PAK5 catalytic activity is mandatory for CE to occur.

X-PAK5/EN mutant affects the adhesive properties of embryonic cells

We thus decided to further analyze how CE movements are regulated by the catalytic activity of X-PAK5 kinase (Fig. 5). Microinjection in the animal hemisphere of X-PAK5/EN mRNA induces defects not previously observed following X-PAK5 K/R mutant expression. During early cleaving stages, both gfp control and X-PAK5/EN-injected em-

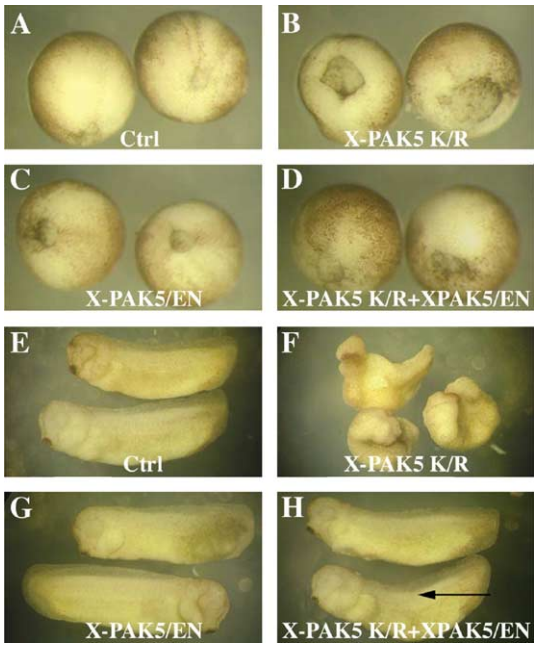


Fig. 4. Rescue of X-PAK5 K/R phenotype by expression of X-PAK5/EN. Four-cell stage embryos were injected in the DMZ either with 300 pg of gfp mRNA or with 300 pg of GFP-X-PAK5 K/R mRNA alone or in combination with 50 pg GFP-X-PAK5/EN mRNA. Stage 12 equivalent embryos (A, B, C, and D). (A) Gfp control (Ctrl) embryos, (B) GFP-X-PAK5 K/R-injected embryos, (C) GFP-X-PAK5/EN-injected embryos, and (D) GFP-X-PAK5 K/R and X-PAK5/EN co-injected embryos. Expression of GFP-X-PAK5/EN efficiently rescues the delay in blastopore closure induced by GFP-X-PAK5 K/R. Stage 28 equivalent embryos (E, F, G, and H). (E) Gpf control (Ctrl) embryos, (F) GFP-X-PAK5 K/R-injected embryos, (G) X-PAK5/EN-injected embryos, and (H) X-PAK5 K/R and X-PAK5/EN co-injected embryos. Expression of GFP-X-PAK5/EN efficiently rescues the phenotype induced by GFP-X-PAK5 K/R as co-injected embryos develop normally or with a slight bent axis as indicated by black arrow.

bryos develop normally (data not shown), but from late morula to early blastula stages, those embryos develop ectodermal lesions in a dose-dependant manner. Eighty percent of embryos (n = 76) injected at two cell stage with X-PAK5/EN mRNA (300 pg) present an abnormal pigmentation around the injection site as indicated by arrows in Fig. 5A (compare panels a–b to d–e). At these early stages (6.5–8), prior to the early gastrula transition checkpoint (EGT) when apoptosis is first detected (Howe et al., 1995), we did not observe blastomere fragmentation or embryo blebbing, as it was previously reported following expression of the catalytic domain of subgroup I X-PAK1 (Bisson et al., 2003). By gastrula (stage 10.25), these embryos develop large ectodermal lesions (see arrow in Fig. 5A, panel f), and we could visualize blebbing of embryonic cells (data not shown), indicating that large amounts of kinase activity are deleterious for embryos and will eventually and consequently induce their death before the end of gastrula.

Embryos injected with lower doses of mRNAs (38–150 pg per embryo) developed visible lesions on the animal pole at late blastula/early gastrula stages (panels a–d in Fig. 5B). Analyses of animal caps dissected from control and X-PAK5/EN-expressing embryos revealed that the latter were hollowed compared to controls (as indicated by black arrows in b′–d′ in Fig. 5B), suggesting that most X-PAK5/EN-expressing cells lost their adhesion to their neighbors and dissociated from the remaining ectodermal layer. Similar ectodermal lesions were previously reported to be induced following expression of IQGAP1 and a E-cadherin dominant-negative mutant that both affect cell adhesion (Levine et al., 1994; Sokol et al., 2001). Loss of cell adhesion induced by expression of X-PAK5/EN mutant was confirmed by performing hemisections of whole embryos expressing either the lineage tracer β-galactosidase alone (control) or together with X-PAK5/EN mRNA. While in control embryos, blue cells are tightly associated to the ectodermal mass, X-PAK5/EN blue cells are loosely associated; some of them dissociated from the roof of the blastocoel and fall into the blastocoel cavity (see white arrow in Fig. 5C, panel c). These results suggest that expression of X-PAK5/EN decreases cell–cell adhesion. To further confirm that X-PAK5 kinase activity regulates adhesiveness between blastomeres, we first looked at cell morphology in animal cap explants dissected from embryos injected with either control gfp mRNA (gfp) or with GFP-X-PAK5/EN mRNA (100 pg per embryo) by using a confocal microscopic approach (Fig. 5D). Gfp control signal is present in a diffuse state in the cytoplasm, faintly at cellular junctions (Fig. 5D, Gfp), and in some nuclei (data not shown). The GFP-X-PAK5/EN signal was essentially diffuse in the cytoplasm and to some extent could be observed in the vicinity of the cytoplasmic membrane. Strikingly X-PAK5/EN-expressing cells appear very loosely associated as depicted in the two images resulting from consecutive 10-μm stacks (Fig. 5D, X-PAK5/EN). These observations strengthen our previous results (Figs. 5A–C) showing that, at the cellular level, catalytic X-PAK5 activity induces a loss of adhesion between embryonic cells.

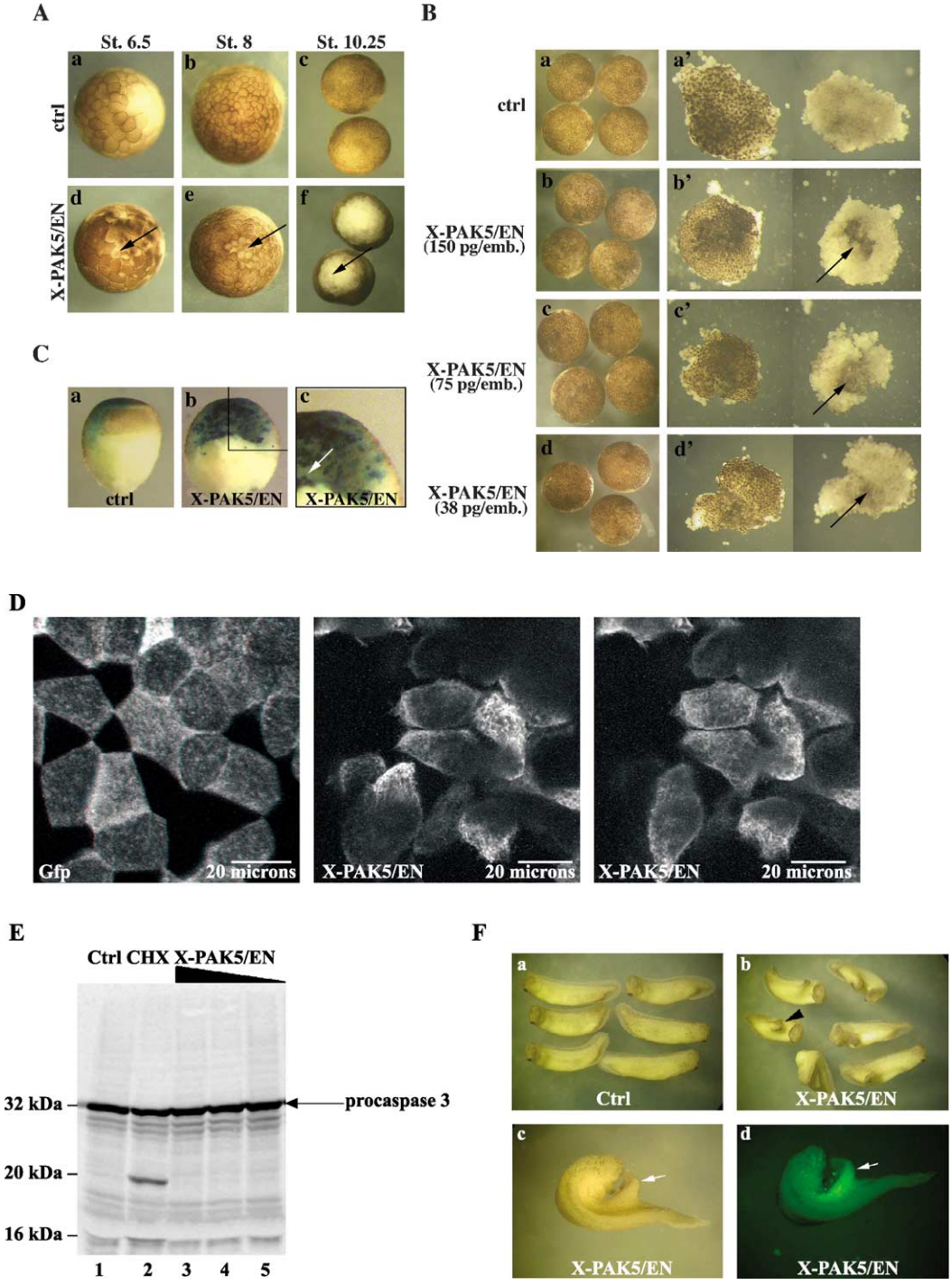
Loss of cell adhesion was previously shown to be induced upon expression of Rac, Cdc42, and Rnd1 GTPases (Choi and Han, 2002; Hens et al., 2002; Wunnenberg-Stapleton et al., 1999). Loss of cell adhesion may also result and thus be a consequence of apoptosis induction (Trindade

Table 2
Rescue of X-PAK5 K/R phenotypes by X-PAK5/EN

mRNA injected	n	Blastopore		Late phenotypes	
		Normal	Open	Normal	Shortened axis
X-PAK5/EN	70	88	12	80	20
X-PAK5 K/R	80	26	74	38	62
X-PAK5 K/R + X-PAK5/EN	72	80	20	70	30

Embryos were injected at four-cell stage in the DMZ with indicated mRNAs. Blastopore closure and late phenotypes were scored at stage 12 and 28, respectively. Numbers refer to percentages of phenotypes, n refers to the total number of embryos analyzed.

et al., 2003). Alternatively, it was previously shown that adhesive properties, motility, and cell fate of prospective head mesodermal cells are affected by blocking PDGFR signaling during gastrulation (Ataliotis et al., 1995; Symes and Mercola, 1996; Van Stry et al., 2004). Interfering with this pathway induces gastrulation defects independent of CE movements and accumulation of mesodermal cells in the blastocoel cavity (Van Stry et al., 2004). These cells are fated to die. However, rescue of apoptosis is not sufficient to restore their motility, indicating that changes in motility and adhesive properties are not a consequence of apoptosis induction. To discriminate between these regulations and since both mammalian and *Xenopus* PAKs were shown to regulate programmed cell death (Bokoch, 2003; Faure et al., 1997), we investigated whether X-PAK5/EN may induce apoptosis in early embryos. We assayed extracts of X-PAK5/EN-injected embryos for caspase activity by testing their ability to induce cleavage of the mammalian procaspase 3 (Fig. 5E). Such assay was previously developed in *Xenopus* embryos using the caspase substrate poly(ADP)ri-



bose polymerase (tPARP) (Hensey and Gautier, 1997; Trindade et al., 2003). In cycloheximide-treated embryo extracts, procaspase cleavage was induced, as visualized by the appearance of proteolytic fragments of 20 and 16 kDa (Fig. 5E, lane 2), but no cleavage was observed in control embryo extracts (lane 1) and in X-PAK5/EN-injected embryos (38–150 pg mRNA per embryo) (lanes 3–5). In addition, expression of mRNA encoding for BCL-XL together with various amounts of X-PAK5/EN mRNA did not prevent the ability of X-PAK5/EN to induce ectodermal lesions and deadhesion of cells, suggesting that, in X-PAK5/EN-injected embryos (38–150 pg per embryo), the decrease in cell–cell adhesion does not result from the induction of the apoptotic program (data not shown). Since these loosely associated cells were not programmed for death, we analyzed what would be their fate in developing embryos. Gfp (controls) or X-PAK5/EN-injected embryos (150 pg mRNA per embryo) were kept growing up to stage 28 and their morphology assessed by direct observation (Fig. 5F). Around 40% of X-PAK5/EN-expressing embryos repeatedly developed very specific and homogenous defects (panel b) compared to controls (panel a). Those defects are characterized by the growth of tissue protruding from the body axis of the embryo (panels b and c). Indeed, cells in these tissues are gfp positive (Fig. 5E, see white arrows in panel d), further proving that body extensions are derived from cells that received the injected mRNA and that injected cells are still alive at this stage of development. It is thus likely that changes in adhesive properties of X-PAK5/EN-expressing cells drive them in inappropriate locations where they participate in the formation of protrusions. These results further confirm that the level of X-PAK5 activity that we expressed in embryos throughout this study does not induce an apoptotic process, but rather a change in adhesive behavior of the cells.

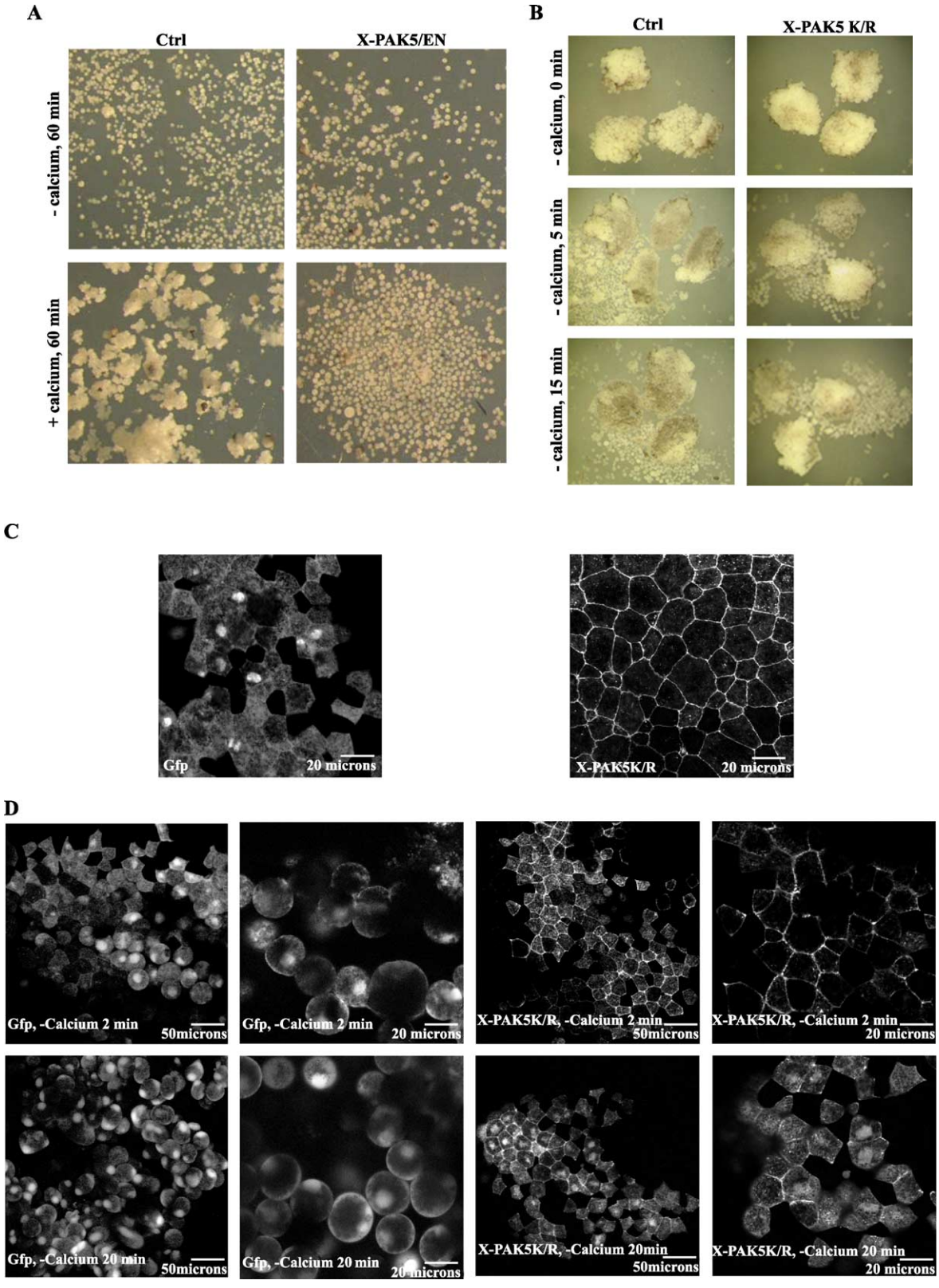
Calcium-dependant cell–cell adhesion is regulated by X-PAK5

To further investigate whether the catalytic activity of X-PAK5 regulates the adhesive properties of embryonic cells, we performed an in vitro aggregation assay (Fig. 6). In this assay, cells from animal explants were dissected at stage 8.5 from control or X-PAK5 mutant-injected embryos. Then, cells were dissociated in Ca^{2+}/Mg^{2+}-free medium and allowed to reaggregate by adding calcium back to the medium. We first analyzed the effect of X-PAK5/EN expression on dissociation and reassociation of cells (Fig. 6A). Cells from both gfp control and X-PAK5/EN-injected cap explants were totally dissociated 60 min after calcium withdrawal (Fig. 6A, −calcium, 60 min). Control cells reaggregated into tightly adherent structures (clumps) an hour after supplementing medium with calcium (Fig. 6A, +calcium, 60 min). In contrast, cells from X-PAK5/EN-injected explants that dissociated more rapidly than control cells (data not shown) either remain isolated or form small loosely associated structures an hour after adding back calcium to the medium (Fig. 6A, +calcium, 60 min). Thus, expression of catalytically active X-PAK5 kinase decreases calcium-dependent cell reaggregation.

We then studied the effect of X-PAK5 K/R expression during the dissociation and reassociation procedure (Figs. 6B, 6C, and 6D). The properties of animal cap cells to dissociate were again directly observed at the macroscopic level and also at the microscopic level. For the latter, we followed the gfp control or GFP-X-PAK5 K/R-expressing cells using a confocal microscope. At the time dissections were performed (−calcium, 0 min), X-PAK5 K/R-expressing cells of the animal caps looked more tightly bound than cells in control caps (Fig. 6B). At the onset of dissociation in calcium-free medium (−calcium, 5 min and

Fig. 5. Expression of X-PAK5/EN induces ectodermal lesions when injected in the animal pole. (A) Two-cell stage embryos were injected in the animal pole either with gfp mRNA or with X-PAK5/EN mRNA (300 pg per embryo). (a–c) Examination of gfp control (Ctrl) embryos. (d–f) Examination of GFP-X-PAK5/EN-injected embryos. (a and d) Stage 6.5, (b and e) stage 8, and (c and d) stage 10.25. X-PAK5/EN-injected embryos exhibit an abnormal pigmentation at the level of injection site (see arrows in d and e) and display large ectodermal lesions visible on the animal pole at gastrula (see arrow in f). (B) Examination of whole embryos and dissected animal caps following expression of various amounts of GFP-X-PAK5/EN mRNA. Uninjected control (Ctrl) embryos (a) or embryos injected at two-cell stage in the animal pole with either 150 pg (b), 75 pg (c), or 38 pg (d) of GFP-X-PAK5/EN mRNA were allowed to develop and animal caps were dissected at early gastrula stage. (a′–d′) Direct examination of control (Ctrl) animal caps (a′) or animal caps dissected from embryos in b (b′), in c (c′), and in d (d′). Black arrows in b′, c′, and d′ indicate the position of the ectodermal lesions. (C) Lineage tracer analyses of GFP-X-PAK5/EN-expressing cells. Two-cell stage embryos were injected in the animal pole either with 200 pg of β-galactosidase mRNA alone or in combination with 200 pg of X-PAK5/EN mRNA. (a, b, and c) β-Gal-stained embryo sectioned at stage 10.25. Control (Ctrl) embryos expressing β-galactosidase mRNAs alone (a). Embryos co-expressing β-galactosidase and GFP-X-PAK5/EN mRNAs (b and c). c represents a higher magnification of b inset. (D) Subcellular localization of GFP-X-PAK5 mutants in embryonic cells. Embryos injected at two cell stage in the animal region with either gfp mRNA (200 pg per embryo) or GFP-X-PAK5 E/N mRNA (100 pg per embryos) were cultured until stage 8.5. Then, animal caps were dissected and gfp signal immediately (without fixation procedure) analyzed with a confocal microscope LSM510. Images result from 10-μm stacks. GFP-XPAK5/EN-expressing cells appear more loosely associated than gfp control-expressing cells. (E–F) X-PAK5/EN does not induce apoptosis. Embryos were injected at two cell stage in the animal region with either gfp mRNA (150 pg per embryo) or with various amounts of GFP-X-PAK5 E/N mRNA. (E) Procaspase 3 cleavage assay on *Xenopus* embryos. Gfp control embryos (Ctrl) (lane 1), uninjected cycloheximide (CHX)-treated embryos (lane 2), or embryos injected with either 150 pg (lane 3), 75 pg (lane 4), or 38 pg (lane 5) of GFP-X-PAK5/EN mRNA. Embryos were harvested at stage 13 and extracts were tested for their ability to induce cleavage of (^{35}S)methionine-labeled mammalian procaspase 3. Only extracts from cycloheximide (CHX)-treated embryos induce procaspase 3 cleavage (visualized by the appearance of proteolytic fragments of 20 and 16 kDa). (F) Examination at late neurula stage of embryos expressing GFP-X-PAK5/EN mRNA (150 pg injected per embryo). Gfp control (Ctrl) (a) or embryos injected with GFP-X-PAK5/EN mRNA (b–d). c represents a higher magnification of X-PAK5/EN-expressing embryo. (d) Direct examination of the gfp signal emitted from the embryo shown in c. X-PAK5/EN gfp positive are located in the protrusion, suggesting that X-PAK5/EN-mislocated cells did not die at the end of gastrulation. White arrows in c and d indicate the position of the gfp-positive protrusion on the embryo.

−calcium, 15 min), we observed a delay in cell dissociation in X-PAK5 K/R-expressing animal cap explants compared to gfp control explants, indicating that X-PAK5 K/R-expressing cells might be more tightly associated than control cells. Eventually, both control and X-PAK5 K/R-expressing caps were dissociated into single cells after 2-h incubation in calcium-free medium (data not shown).



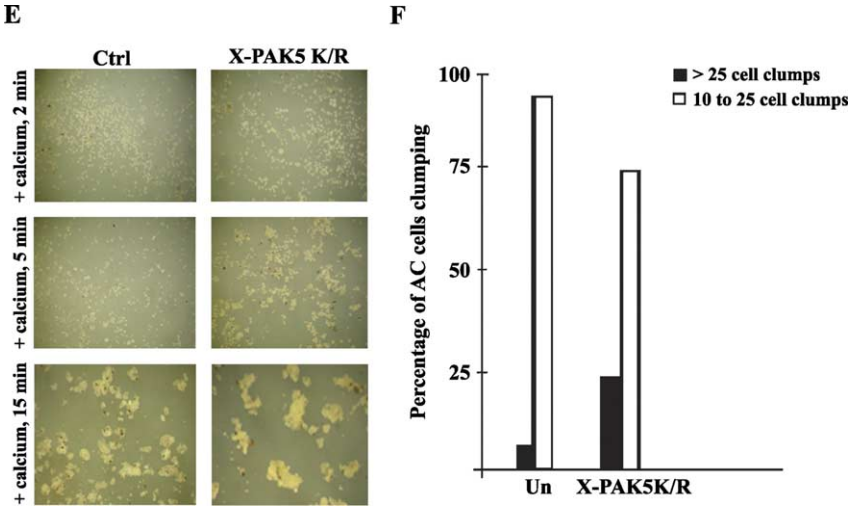


Fig. 6. X-PAK5 catalytic activity regulates the adhesive properties of embryonic cells. (A) Aggregation assay. Two-cell stage embryos were injected in the animal pole either with gfp mRNA (150 pg per embryo) or with GFP-X-PAK5/EN mRNA (150 pg per embryo). Then, animal regions were dissected at stage 8.5, incubated in Ca^{2+}/Mg^{2+}-free medium to allow complete dissociation of cells, and then reaggregated by adding back calcium to the medium. (A) Effect of expression of GFP-X-PAK5/EN on dissociation (upper panel) and reaggregation (bottom panel) of cells. Gfp control cells (Ctrl) were totally dissociated after 1 h in Ca^{2+}/Mg^{2+}-free medium (−calcium, 60 min). Their reassociation was visualized 1 h after calcium addition (+calcium, 60 min). GFP-X-PAK5/EN cells were totally dissociated after 1 h in Ca^{2+}/Mg^{2+}-free medium (−calcium, 60 min). However, GFP-X-PAK5/EN-expressing cells failed to reaggregate when calcium was added back to the medium (+calcium, 60 min). (B) Effect of expression of GFP-X-PAK5 K/R on dissociation of cells. Two-cell stage embryos were injected on the animal pole either with gfp mRNA (150 pg per embryo) or with GFP-X-PAK5/EN mRNA (150 pg per embryo). Gfp control (Ctrl) or GFP-X-PAK5 K/R-injected animal explants were dissected in Ca^{2+}/Mg^{2+}-free medium and immediately harvested (−calcium, 0 min) or harvested after 5 min (−calcium, 5 min) or 15 min (−calcium, 15 min) incubation. GFP-X-PAK5 K/R expression delays dissociation of cells. (C–D) Subcellular localization of GFP-X-PAK5 K/R mutant in animal cap explants. Embryos were injected at two cell stage with either gfp mRNA (200 pg per embryo) or GFP-X-PAK5 K/R mRNA (250 pg per embryo). Animal caps were dissected at stage 8.5 and either directly examined for gfp signal on confocal microscope (C), or incubated in Ca^{2+}/Mg^{2+}-free medium either for 2 min (Gfp, −calcium, 2 min; GFP-X-PAK5 K/R, −calcium, 2 min) or for 20 min (gfp, −calcium, 20 min; X-PAK5/KR, −calcium, 20 min) (D). (E–F) Effect of expression of GFP-X-PAK5 K/R on reaggregation of cells. Gfp control (Ctrl) or GFP-X-PAK5 K/R-injected animal caps were totally dissociated in Ca^{2+}/Mg^{2+}-free medium for 2 h. Then, calcium was added back to the medium and cells were harvested after either 2 min (+calcium, 2 min), 5 min (+calcium, 5 min), or 15 min (+calcium, 15 min), and reaggregation of cells was examined. (F) Graph representing the relative aggregation of animal cells after 15-min incubation with calcium in the representative experiment shown in D. The sizes of the clumps are indicated in legend above the graph.

These results were confirmed at the microscopic level. Before calcium removal, gfp control signal was detected in the nuclei and faintly at cellular junctions (Fig. 6C, Gfp), while strikingly, signal from the gfp-X-PAK-K/R fusion protein concentrated at cell junctions and stained to a lower extent the cytoplasm (Fig. 6C, X-PAK5 K/R). These kinase-dead mutant-expressing cells looked tightly associated. At the onset of dissociation in Ca^{2+}/Mg^{2+}-free medium, animal cap cells expressing gfp control became rounder and a partial loss of adhesion between them was observed (Fig. 6D, Gfp, −calcium, 2 min). These changes in cell shape and cohesion greatly evolved during the 20-min incubation period in calcium-free medium; by that time, most of the cells were completely round and very loosely attached to each other (Fig. 6D, Gfp, −calcium 20 min). The gfp signal appeared delocalized and concentrated to one region of cells. In contrast, the general shape of animal caps expressing GFP-X-PAK5 K/R was not affected after 2-min incubation in Ca^{2+}/Mg^{2+}-free medium (Fig. 6D, X-PAK5 K/R, −calcium 2 min) and the dead-kinase was still present at cell junctions. After 20-min incubation without calcium, GFP-X-PAK5 K/R to some extent still associated to cell–cell junctions, although more cytoplasmic and even nuclear staining of the dead kinase was observed at that time (Fig. 6D, X-PAK5-K/R, −calcium, 20 min). We thus confirm that dead-kinase expression delays the loss of adhesion between embryonic cells that result from the absence of calcium in the medium.

At the macroscopic level, expression of X-PAK5 K/R interfered as well with the kinetics of calcium-dependent cell reaggregation. Indeed, once totally dissociated, X-PAK5 K/R-expressing cells started to associate to each other as soon as 5 min after calcium addition, whereas control cells remained individualized (Fig. 6E, +calcium, 5 min). Later during the reassociation process, clumps formed of X-PAK5 K/R-expressing cells were repeatedly larger than controls clumps (Fig. 6E, X-PAK5-K/R, +calcium, 5 min; +calcium, 15 min; Fig. 6F). Taken together, our results show that X-PAK5 catalytic activity regulates the adhesive properties of embryonic cells in response to extracellular calcium.

Endogenous X-PAK5 activity is regulated in a calcium-dependant manner

Our results indicate that keeping a constant level of X-PAK5 activity in embryonic cells by expressing X-PAK5/

EN both decreases ectodermal cell–cell adhesion in whole embryos and prevents cell–cell reaggregation induced by calcium in an aggregation assay (Figs. 5 and 6). If endogenous X-PAK5 regulates in vivo calcium-dependent cell–cell adhesion during early development, its activity is likely to be finely regulated to allow reassociation of cells. This hypothesis led us to measure the endogenous X-PAK5 activity during the dissociation and reassociation procedure in an aggregation assay (Fig. 7). Animal cap explants from uninjected embryos were dissected at stage 8.5 in Ca^{2+}/Mg^{2+}-free medium and harvested immediately after dissection (−calcium, 0 min) or after incubation of 5 or 15 min (Fig. 7A). Once cells were totally dissociated (−calcium, 60 min), they were allowed to reaggregate by adding calcium back to the medium (Fig. 7B). Both activity and levels of endogenous X-PAK5 were analyzed during the dissociation and reassociation procedure. Endogenous X-PAK5 activity was measured by Western blot analyses using anti-phosphoPAK5 antibodies. These antibodies were made against a phosphopeptide located in the catalytic loop of subgroup II PAKs and surrounding Ser533 in X-PAK5. They specifically detect catalytically active X-PAK5 (see also Materials and methods and Supplementary Fig. S1). During the cells' dissociation procedure (Fig. 7A), no dramatic changes in X-PAK5 activity were observed using anti-phosphoPAK5 antibodies, and X-PAK5 expression level, as well as X-PAK2 and γ-tubulin levels that serve as loading controls, remained constant. However, a very strong inhibition of endogenous kinase activity was observed as soon as calcium was added back to the medium (Fig. 7B). Downregulation of kinase activity was measured 5 min after addition of calcium and while cell aggregation was not yet observed (Fig. 7C, +calcium, 5 min). Inactivation of X-PAK5 was correlated with a delay in its electrophoretic mobility that was analyzed in parallel by Western blot. X-PAK2 and γtubulin levels analyzed in parallel on the same Western blot insured that loading was accurate and that X-PAK5 mobility shift upon calcium addition was specific. We previously show that maintaining a constant level of X-PAK5 activity in embryonic cells prevents cell–cell reaggregation induced by calcium (Fig. 6A). Here, we show that inactivation of endogenous X-PAK5 upon calcium addition precedes reaggregation of cells. Altogether, our data suggest that inactivation of X-PAK5 may be a prerequisite for reaggregation of cells in the presence of calcium. During the course of the reassociation procedure, we observed the reappearance of the phosphoX-PAK5 signal, indicating that the kinase slowly got phosphorylated on residue serine 533 and thus reactivated once cells began to reaggregate (Fig. 7B). Reappearance of the phosphoX-PAK5 signal was correlated at 30 min by its mobility on Western blot, mobility that tended to return to normal (Fig. 7B, compare X-PAK5 and PX-PAK5 panels).

When CE movements occur, cells rapidly change neighbors. This is likely accompanied by discrete and localized changes in extracellular calcium concentration that allow cells to intercalate between each other. In these physiological conditions, individual cells must be in different responsive state regarding adhesion/loss of adhesion to their neighbors. We indeed could not mimic such precise events to address the regulation of X-PAK5 catalytic activity upon changes in extracellular calcium. Nonetheless, we show that in an assay in which all cells are dissociated, the addition of exogenous calcium downregulates the endogenous X-PAK5 catalytic activity. This result clearly indicates that endogenous X-PAK5 activity

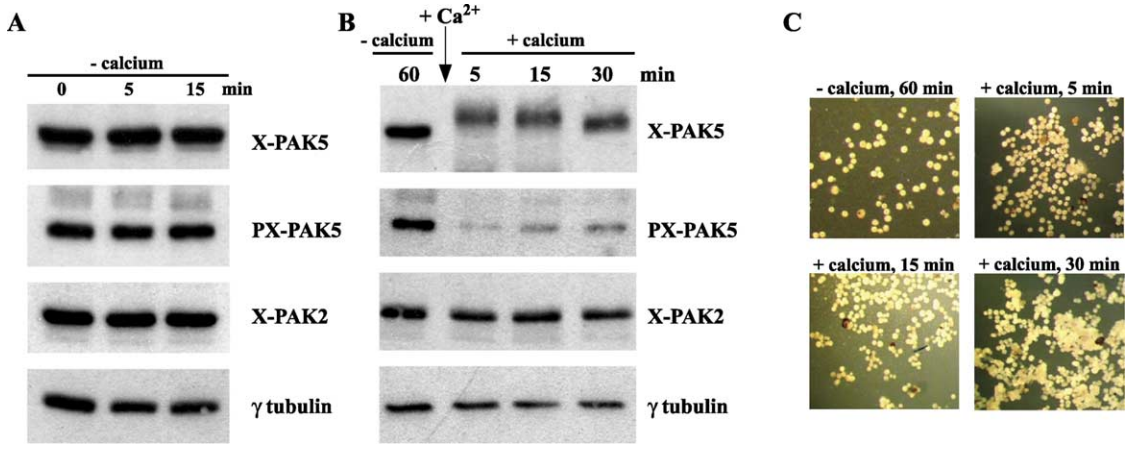


Fig. 7. Endogenous X-PAK5 kinase activity is regulated by a calcium-dependent event. Stage 8.5 uninjected embryos were transferred to Ca^{2+}/Mg^{2+}-free medium and animal caps dissected and harvested either immediately after dissection (−calcium, 0 min) or during the dissociation procedure after 5 min (−calcium, 5 min), 15 min (−calcium, 15 min), or 60 min (−calcium, 60 min) of incubation. Then calcium was added back to the medium and cells were harvested during the reassociation procedure after 5 min (+calcium, 5 min), 15 min (+calcium, 15 min), or 30 min (+calcium, 30 min) of incubation with calcium. (A) Dissociation procedure. (B) Reassociation procedure. In A and B, lysates of explants were analyzed for endogenous X-PAK5 activity by Western blot analysis using anti-PhosphoPAK5 antibodies that specifically recognize catalytically active Ser533-phosphorylated X-PAK5 (also see Materials and methods). In parallel, X-PAK5, X-PAK2, and γ tubulin levels were analyzed by Western blot. (C) Examination of cells during the reassociation procedure shown in B.

could be regulated during the association/dissociation switches that embryonic cells must undergo during CE movements.

X-PAK5 is located at cell–cell junctions and partially colocalizes with adherens junction proteins

We showed that the catalytic activity of X-PAK5 regulates adhesion between cells (Figs. 5 and 6) and that endogenous X-PAK5 activity is downregulated by calcium during the reassociation procedure (Fig. 7B). Cadherins are major transmembrane proteins that are complexed with catenins and mediate calcium dependent cell–cell adhesion (for review, see Gumbiner, 2000). We thus decided to examine whether endogenous X-PAK5 and overexpressed mutants may colocalize with known adherens junctions proteins. We used A6 epithelial cells that were either mock transfected and stained for endogenous PAK5 or transfected by GFP-X-PAK5-K/R or GFP-X-PAK5/EN. All cells were immunostained with either γ catenin or E-cadherin antibodies (Fig. 8). In almost confluent monolayers, endogenous X-PAK5 is present both in a punctate pattern in the cytoplasm and at cell–cell junctions where it colocalizes with both E-cadherin and γ-catenin (upper panel in Fig. 8). However, colocalization at cell junctions is not total, suggesting that protein recruitment at the junction may be regulated. In X-PAK5-K/R-expressing cells (medium panel), the gfp signal detected shows that the kinase strongly associates to modified microtubules as previously reported (Cau et al., 2001), but stains as well the cytoplasmic membrane where it colocalizes at cell junctions with both γ-catenin and E-cadherin (see arrows). Cells expressing active kinase (X-PAK5/EN, bottom panel) have a very altered morphology (Cau et al., 2001) presenting long cytoplasmic extensions and are growing above the cell monolayer (as seen with Z projection on confocal microscope, data not shown). In these cells, E-cadherin is still present at the plasma membrane and also in the cytoplasm. The gfp signal detected shows that the active kinase is essentially diffuse in the cytoplasm and does neither appear enriched at the cytoplasmic membrane nor colocalized with E-cadherin. However, in cells expressing lower levels of active kinase that are still part of the monolayer, colocalization between X-PAK5/EN and E-cadherin was often observed (data not shown). Such colocalization is also observed with γ-catenin (X-PAK5/EN and γ-catenin panels) where the end of processes in expressing cells that contact cells in the monolayer are costained with both X-PAK5/EN and γ-catenin (see arrows). In summary, localizations of the endogenous X-PAK5 and the dead and active exogenous mutants are consistent with X-PAK5 function in regulating adhesion between cells.

Discussion

The formation and elongation of the embryonic body axis during *Xenopus* gastrulation are triggered by cellular rearrangements commonly termed convergent extension (CE) (Wallingford et al., 2002). CE movements are accompanied by changes in shape, polarity, and adhesive

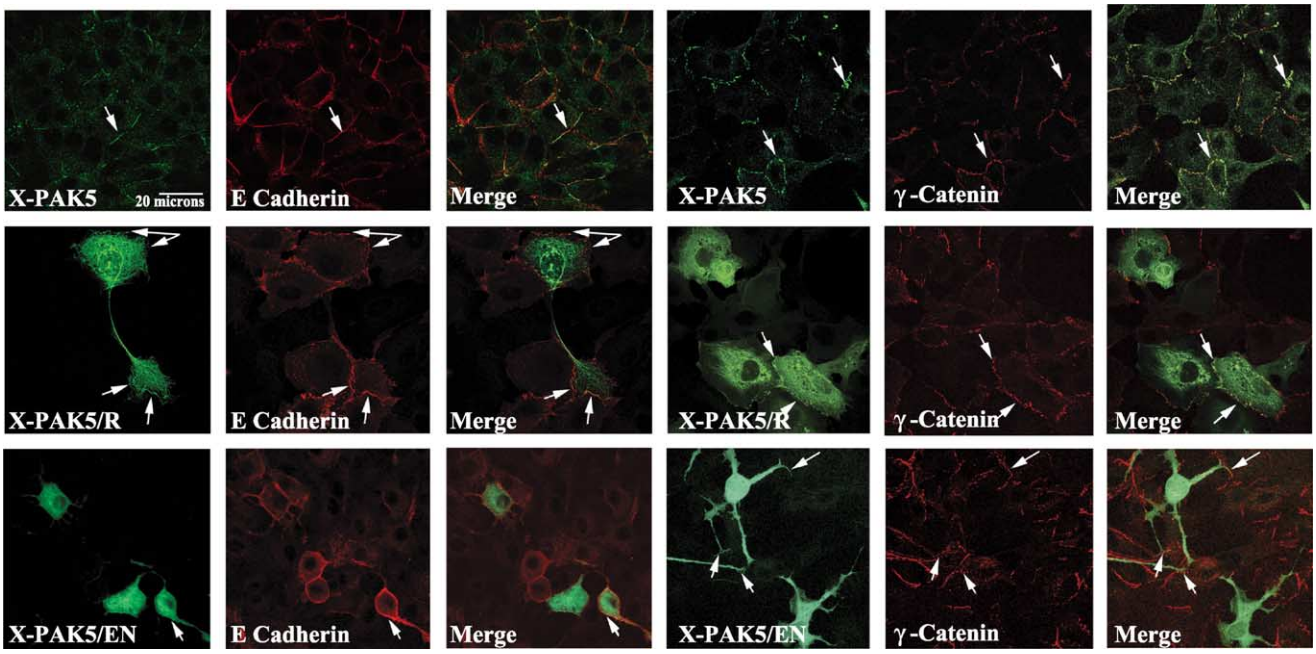


Fig. 8. Localization of endogenous X-PAK5, X-PAK5K/R, and X-PAK5/EN mutant epithelial A6 *Xenopus* cells. Almost confluent A6 epithelial cells were mock, GFP-X-PAK5/KR, or GFP-X-PAK5 E/N transfected for 24 h. Colocalization of the endogenous X-PAK5, dead, and active kinase with γ-catenin and E-cadherin was performed.

properties of cells. During these rearrangements, cells of the marginal zone elongate and extend mediolaterally polarized filopodia and lamellipodia; these protrusions make stable contacts with neighboring cells and exert traction permitting cells to intercalate (Keller et al., 2000; Shih and Keller, 1992; Tahinci and Symes, 2003). Although the morphological details of CE movements during gastrulation in *Xenopus* embryos are well characterized, the molecular basis of these cell rearrangements remains to be clarified.

PAKs are multifunctional kinases that play key functions in cell cytoskeleton rearrangement, polarization, and migration (for review, see Bokoch, 2003; Jaffer and Chernoff, 2002). We previously reported the identification of X-PAK5, a *Xenopus* PAK protein that is involved in the regulation of cytoskeleton organization (Cau et al., 2001). Indeed, in cell culture ,the kinase-dead mutant stabilizes a subset of MTs, while active kinase induces major cell shape changes, characterized by cytoplasmic processes that are decorated by filopodia (Cau et al., 2001). These properties led us to investigate the putative roles of X-PAK5 in *Xenopus* embryo morphogenesis.

X-PAK5 activity regulates adhesion between cells during CE movements

In this study, we show that X-PAK5 may play an important role in regulating *Xenopus* morphogenesis through its ability to modulate cell adhesive properties. First, X-PAK5 is expressed in tissues undergoing extensive morphogenetic movements at gastrula such as the ectoderm and the involuting mesoderm (Fig. 1). Similar spatial distribution was reported for genes whose roles have been suggested in morphogenesis. Most of these genes encode either for cell adhesion molecules such as cadherins (Costa et al., 1998; Kuhl et al., 1996; Lee and Gumbiner, 1995; Levine et al., 1994), integrins (Ramos et al., 1996), and protocadherins (Bradley et al., 1998; Kim et al., 1998), or for proteins that regulate cell adhesion including small Rho-like GTPase XRnd1 (Wunnenberg-Stapleton et al., 1999), XRac, and XCdc42 (Choi and Han, 2002; Lucas et al., 2002) that bind both X-PAK5 (Cau et al., 2001) and components of the noncanonical Wnt signaling pathway such as XWnt-5a (Moon et al., 1993) and XFz7 (Djiane et al., 2000). Second, expression of X-PAK5 kinase activity in developing embryos causes a dramatic loss of cell adhesion (Figs. 5A–D) while embryonic cells expressing the dead kinase are tightly associated together. The dead kinase being sharply localized at the cells boundaries (Fig. 6C). These results indeed suggest that X-PAK5 activity may be involved in regulating cell–cell adhesion. Third, the endogenous X-PAK5 is localized at the adherence junctions in animal cap cells and in DMZ cells (Fig. 1D). Fourth, endogenous kinase activity (Fig. 7) and localization (not shown) are not modified upon embryonic cell dissociation by calcium removal. However, its activity is downregulated when calcium is added to the medium. X-PAK5 inactivation occurs before newly formed junctions between cells become visible (data not shown), indicating that downregulation of kinase activity may be required for the establishment of new junctions. This regulation likely represents a transient response of the kinase to calcium since once cell junctions clearly form, the kinase slowly got reactivated. This is confirmed by an assay showing that calcium addition to dissociated cells is no longer efficient to induce adhesion of cells when X-PAK5 is kept catalytically active while adhesion is facilitated in cells expressing the dead kinase mutant (Figs. 6E–F). Moreover, dead kinase (KR mutant) expression in embryonic cells delays their dissociation when calcium is removed from the extracellular medium (Figs. 6B and D). Thus, X-PAK5 may regulate adhesion between cells by interfering with the calcium response.

Finally, expression of a kinase-dead mutant of X-PAK5 inhibits dorsal cell intercalation during CE movements in whole embryos and in activin-treated animal cap explants without affecting mesodermal specification (Figs. 2 and 3). The involution of marginal zone cells during gastrula requires the loosening and rearrangements of cell–cell contacts (Wilson and Keller, 1991). In embryos expressing X-PAK5/EN in the DMZ, CE movements are impaired as a consequence of cell dissociation. We speculate that, in X-PAK5 K/R-expressing embryos, the adhesiveness of expressing cells is too strong and prevents the dynamic cell rearrangements necessary for CE. Thus, a very tight balance is likely to exist between activation/inactivation of the X-PAK5 catalytic activity in order to regulate CE.

At this point it is tempting to speculate that activation/inactivation switches of X-PAK5 may be regulated by the intercellular waves of calcium that were previously described in tissues undergoing CE (Gilland et al., 1999; Leclerc et al., 2000). More specifically, in *Xenopus* DMZ explants, transient calcium waves are required for CE movements to occur (Wallingford et al., 2001) and the authors proposed they may represent a general mechanism to coordinate CE. The relationship between X-PAK5 and these intercellular calcium waves would indeed be an interesting issue to address in the future. Taken altogether, our results suggest that the function of X-PAK5 in the embryo is to regulate adhesion of cells that are undergoing morphogenetic movements under intercellular calcium signaling.

PAKs also play active functions during epithelial cell colony spreading and dissociation in response to HGF (Royal et al., 2000), a model that implies as well deadhesion and dissociation of cells before motility can occur. Finally, hPAK4, another subgroup II PAK member, alters cell adhesion and increases invasiveness potential (Qu et al., 2001). We cannot rule out in our study that X-PAK5/EN and X-PAK5K/R may interfere with the activity of another PAK family member. This hypothesis remains, however, unlikely since endogenous X-PAK5 is present at cell junctions as well and is regulated when dissociated embryonic cells from cap explant are allowed to associate by calcium addition. Indeed, other members of the *Xenopus* PAK gene family are

expressed during development but their patterns are clearly distinct from that of X-PAK5 (Islam et al., 2000; Souopgui et al., 2002) and neither X-PAK1 nor X-PAK3 appears to regulate cell adhesion (Bisson et al., 2003; Souopgui et al., 2002).

Loss of adhesion between cells is sometimes a consequence of induced apoptosis, and PAK members play active functions in regulating (both positively and negatively) the apoptosis pathway. For example, X-PAK1 has protective function against apoptosis in *Xenopus* egg extracts (Faure et al., 1997) while its activation by proteolytic cleavage is sufficient for apoptotic body formation during *Xenopus* early development (Bisson et al., 2003). Although the lesions we observed with expression of active X-PAK5 mutant were clearly different than X-PAK1-induced lesions (Bisson et al., 2003), we studied the fate of active kinase-expressing cells to eliminate the hypothesis that control of cell–cell adhesion by PAK5 was a mere consequence of apoptosis induction. Tadpole-expressing active X-PAK5 does not die, nor the X-PAK5 expressing cells that also multiply and form tissue protrusion on tadpole bodies. These protrusions likely emanate from cells that lost their adhesion and fall on the floor of the blastocoel.

Which factors regulate X-PAK5 activity?

During CE movements, rapid changes are likely to be required at cell/cell junctions to remodel the adhesive contacts that must break and form again (Gumbiner, 2000) and to regulate protrusive activity. We hypothesize that a switch between activation/inactivation of the X-PAK5 catalytic activity may participate to such regulation.

The small GTPase Cdc42 regulates Ca^{2+}-dependent cell adhesion in *Xenopus* embryonic cells. IQGAP1, a Cdc42 effector, regulates cell adhesion in response to calcium and induces similar ectodermic lesions than X-PAK5 during early development (Sokol et al., 2001). In addition, Rac, RhoA, and XRnd1 GTPases are also reported to regulate cell adhesion in the ectoderm (Hens et al., 2002; Wunnenberg-Stapleton et al., 1999). During CE, protrusive activity is at least modulated by Rac and RhoA GTPases (Tahinci and Symes, 2003). They regulate lamellipodia and filopodia formation (rac-dependent), polarization of the protrusive activity (Rac and Rho-dependent) and cell shape (Rho-dependent) (Tahinci and Symes, 2003). Such a study on Cdc42 functional importance in regulating protrusive activity during CE is still lacking and would be of interest. Active X-PAK5 promotes filopodia in cell culture (Cau et al., 2001), and we did observe that expression of low X-PAK5 activity in animal cap explant cells induced a protrusive activity mainly characterized by filopodia (data not shown). In the future, these observations indeed will require to be more documented by dynamic studies in gastrula embryos. Thus, X-PAK5 activity may be regulated by one these GTPases or a yet nonidentified member of this family. However, X-PAK5 binding both to Rac or Cdc42 GTPases does not allow its catalytic activation (Cau et al., 2001), a common feature to subgroup II PAK members. Therefore, alternate pathways that involve complex recruitment to the plasma membrane as recently described for subgroup I PAK should also be considered (Li et al., 2003).

We present preliminary data in Fig. 8 that show endogenous X-PAK5 and the kinase active and kinase dead ectopic X-PAK5 colocalization with the two adherens junction proteins E-cadherin and γ-catenin. Endogenous kinase partial colocalizations with E-cadherin and γ-catenin indicate that its association to cell–cell junctions may be regulated. Both mutants also share some colocalization with these two markers. Indeed, more partners of the adherens junctions should be studied for their colocalization with endogenous X-PAK5 and their putative mislocalization under the expression of active and dead PAK5 kinase mutants (see below). This work is currently under development in the laboratory.

It is tempting to speculate that X-PAK5 activation/inactivation switch may control the assembly of a membrane-bound complex that would form at cell–cell junctions and control adhesion. In such complex, partners would likely evolve after modification (i.e., phosphorylation). Cadherins are key components of adherence junctions (for review, see Aplin et al., 1999). Many other proteins such as Dishevelled, PKC, Rho, Rac, Cdc42 and IQGAP, p120 catenin, and α-catenin are also located at cell junctions and could potentially regulate X-PAK5 activity. IQGAP associates with filopodia in *Xenopus* cells (Yamashiro et al., 2003) and induces their formation (Swart-Mataraza et al., 2002). Translocation of IQGAP1 to cell–cell contacts disrupts cadherin-catenin complexes and decreases cell–cell adhesion (Kuroda et al., 1998; Li et al., 1999). IQGAP1-induced lesions in the ectoderm of *Xenopus* embryo depend upon its capacity to bind Cdc42, although the latter is not sufficient to confer that phenotype (Sokol et al., 2001), suggesting that another factor is required. In that regard, it will be of specific interest in the future to study whether PAK5 may participate to IQGAP1-induced phenotype in adhesion or if Cdc42-bound IQGAP1 may modulate X-PAK5 activity.

We speculate that p120 catenin, a cadherin binding protein and a member of the armadillo protein family, could be another putative target for X-PAK5 kinase. Indeed, p120 catenin mutants regulate cell adhesion and can activate Rac and Cdc42 GTPases (for review, see Gumbiner, 2000). The p120 catenin protein was recently shown to bind microtubules and promote the traffic of cadherin to the cell surface (Chen et al., 2003). Interestingly, mutation of serine residues to alanine in the Arm repeat of p120 catenin promotes its association with interphase microtubules (Franz and Ridley, 2004). These interphase microtubules are stabilized and look like X-PAK5K/R-induced stable microtubules. Moreover, the wild-type p120 catenin induces a dendritic morphology that resembles much of X-PAK5/EN-induced changes in cell morphology. Relationships

between X-PAK5 and p120 catenin represent an interesting issue to study in the future.

In conclusion, this study identifies X-PAK5 as a new regulator of CE movements during gastrulation. Future studies to address the mechanism by which X-PAK5 regulates CE movements will include the examination of its function in recruitment or phosphorylation of other cell junction proteins at the plasma membrane.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.10.005.

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